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PROTECTIVE INOCULATION AGAINST ASIATIC CHOLERA.*

AN EXPERIMENTAL STUDY.

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THE epidemic of Asiatic cholera which has recently passed through the Philippine Islands has brought forcibly before us the particular difficulties encountered in combating and controlling a disease of this nature in a tropical country and among a partly uneducated people. Moreover, its history has demonstrated the impossibility of eradicating or even of satisfactorily controlling the malady in this city by ordinary hygienic methods, that is, by those measures directed solely towards the purification of the food and water supply of the infected districts. During the period at which the number of infected individuals was the greatest, it was shown by studies made in this laboratory that, in Manila at least, the disease was not usually transmitted directly by water,[2] but more often by food infection. Under the conditions existing here, an individual suffering with cholera or convalescent from it might frequently infect the food of healthy persons. Although the Board of Health took every precaution practicable with regard to the furnishing of uninfected water, the prohibition of the sale of many fruits and other raw food material, and the isolation and treatment of the sick, nevertheless the epidemic spread, continued for nearly two years, and caused the death of 3,866 people in the city of Manila alone. Hence, it was evident,

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* Received for publication September 15, 1904.

[1] The experimental work which forms the basis of this article was for the greater part performed during the spring of 1903 in the "Institut für Infektionskrankheiten," Berlin (Professor R. Koch, Director, Department of Professor A. Wassermann). I wish to express here my very grateful thanks to Professor A. Wassermann, under whose direction the research was first undertaken, for many suggestions and courtesies during the course of my studies.

[2] In other portions of the Archipelago the disease was certainly conveyed and spread by the water supply.

at least with a population of this character, that since it was not possible to prevent many individuals from coming into contact with and even ingesting the cholera organism, it would be advisable to immunize them artificially and to protect them by vaccination against the disease.

HISTORICAL.

The methods which have been employed for human protective inoculation against cholera are not numerous. Ferran, in an epidemic which raged in Spain, was the first to introduce the vaccination of human beings against the disease. Several government commissions were appointed to investigate Ferran's method, but their opinions as to its merits were for the most part unfavorable. Gamaleia then suggested the use of sterilized virulent cultures for the injection, but Haffkine returned to the use of living cultures, making a first inoculation from a standard attenuated culture and using a virulent culture for a later treatment. Afterwards, following Tamancheff, Haffkine recommended the addition of a 0.5% solution of carbolic acid to the culture. Kolle maintainted that a single injection of living or dead cholera vibrios gave as good an immunity as did the repetition of the dose. Patients whose serum normally had a value of 0.75-0.6, showed ten days after inoculation a serum value of .003 grms., *i. e.*, 0.003 grms., protected guinea pigs against ten times the fatal dose of cholera. Kolle recommended the following method: a well-grown agar culture containing about 20 mg. of growth is emulsified in 10 c.c. of physiological salt solution, and sterilized for a few minutes at 50° C.; 0.5% phenol may be added. In vaccinating, one c.c., equal to two mg. of the culture, is injected subcutaneously. Larger amounts were occasionally employed by Kolle.

Several investigators, Klemperer, Ketscher, and Sawtchenko and Sabotolny, have endeavored to produce immunity against cholera by feeding experiments, but this method, being tedious and uncertain, has obtained no practical acceptance. Various vaccines have also been prepared, some of them chemically, by Brieger and Wassermann, Federoff, Wassermann, Klebs, Rosminski; Issaeff attempted to increase natural resistance by inducing phagocytosis; Buchner and Hahn extracted by pressure a so-called cholera plasmin which was used for inoculation; Emmerich and Loew asserted the efficiency of "pyocyanase" against the cholera vibrio; and finally Besredka used for inoculation organisms which had been saturated with immune serum.

Passive immunization against Asiatic cholera by the use of antitoxic or bactericidal sera need not be discussed here, as it is obvious, because of the brief immunity which such sera confer, that their value as a practical prophylactic is very limited.

From this review it is evident that as yet no satisfactory form of protective inoculation against cholera has been established. Usually those prophylactics which give rise to the necessary immunity produce too severe a local reaction; while no other, causing but a mild local reaction, has proved sufficiently protective. Of the methods employed, those of Haffkine and of Kolle promise the best results, but a few preliminary trials with Haffkine's method showed its impracticability for these Islands; first because, on account of the severe local and general reaction, the natives would not voluntarily submit to it; secondly, because on account of this violent reaction and of the unsettled condition of the country, it was inadvisable to make such vaccination compulsory; lastly, because, while this inoculation gives rise to a bactericidal and agglutinative serum in the inoculated, the antitoxic value of such serum is probably very slight.

On account of the great importance of this question, an experimental study was undertaken with the object of obtaining some practicable and efficacious form of inoculation against the disease.

DESCRIPTION OF THE CULTURES.

In my search for practical vaccine, I first studied the local reaction and other toxic effects produced in animals after the injection both of a very virulent cholera culture and of one which, through long cultivation upon artificial media, had lost most of its virulence. The effects of the killed as well as of the living organisms were also studied with each culture. These two races, for the sake of brevity, will be referred to in this article as "Virulent" and "Avirulent."

The avirulent organism was obtained through the kindness of Professor Wassermann. It had been isolated by R. Pfeiffer in the epidemic of cholera which occurred in Hamburg in 1894, and preserved for nine years on artificial media, with passage through animals from time to time. During the preceding year, however, the strain employed in these experiments had been grown only on artificial media, and not inoculated into animals. Its growth on all culture media was typical for Spirillum choleræ asiaticæ, including the production of nitroso-indol in peptone solution. In its morphological characteristics it was not typical, in that short commas were seldom observed, but instead rather long threadlike forms; also its motility was lost to a great extent, though not entirely. However, it was agglutinated by a standard cholera immune serum in even higher dilutions than was another genuine cholera strain; and there is no doubt of its representing a true though attenuated type of Spirillum choleræ asiaticæ.

The virulent organism was isolated by Professor Kolle in Jaffa during the recent epidemic of cholera there. It reacted typically in all media, and its morphology and motility were also characteristic of the genuine cholera organism. It was agglutinated in high dilutions by the cholera immune serum above mentioned, though in less degree than the avirulent strain.

These cultures were accurately standardized, and the minimal lethal dose for guinea pigs of 250 grams' weight determined. After numerous passages of "Virulent" through animals, a lethal dose of 0.1 of a standard loop[1] (two mg.) of a 20-hour agar culture was reached. Such a dose of "Virulent," when suspended in one c.c. of a 0.85% NaCl solution and injected intraperitoneally into a guinea pig of 250 grams' weight, regularly caused death within 24 hours. With "Avirulent," on the other hand, 1.5 standard loop of a 20-hour agar culture, injected intraperitoneally, was required to produce death within the same time in such an animal. The former culture, therefore, may be said to possess 15 times the virulence of the latter. Throughout the course of the work this relationship between the two organisms was carefully preserved and continually tested by inoculation, a precaution especially necessary because of the rapid change in virulence of the cholera spirilla when grown on laboratory media.

METHOD.

The reactions for agglutination were performed in the test-tube. One loop of the living organisms was thoroughly emulsified with one c.c. of an 0.85% NaCl solution. The diluted serum, suspended in one c.c. of a similar salt solution, was then added, the tube well shaken, and the mixture allowed to stand for two hours at 37° C. In a complete agglutination the liquid overlying the precipitated bacteria is entirely clear; in a weak reaction, although there is a distinct agglutination, with a precipitation visible to the naked eye, the supernatant fluid remains more or less cloudy.

The bactericidal reactions were tested in the abdominal cavity of guinea pigs according to the method of R. Pfeiffer, a syringe with a blunt-pointed needle being employed. Care was taken to avoid injury to the intestine. The dilutions of the serum were made in 0.85% NaCl solution. One cubic centimeter of the diluted serum was then added to one c.c. of broth containing two loops of "Virulent" in suspension, after which one c.c. of the resulting mixture was injected into the peritoneal cavity of a guinea pig of 250 grams' weight (or a little less), the animal thus receiving 10 times the fatal dose of the living organisms. A fresh guinea pig was of course used for each reaction. The experiment was controlled by microscopic examination of a drop of serum from the abdominal cavity, obtained by means of a capillary tube; this was taken immediately and again 20 minutes after the inoculation. Control animals were also inoculated with 10 times the fatal dose of "Virulent," but without serum. The result to the animal after 24 hours, whether it was then living or dead, was regarded as the final test, though the condition of the organisms in the abdominal cavity after 20 minutes was always carefully noted.

It soon became evident that the local reaction upon the tissues after a subcutaneous injection of "Avirulent" was much less than after one with the virulent cholera spirillum. I therefore decided to determine the character of the serum which could be produced with this avirulent organism and to compare it with that produced by the virulent germ.

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[1] This standard loop was employed throughout the work.

Rabbit series No. I.—A series of six rabbits of an average weight of 1,500 grams were inoculated intravenously, three each with one-half loop of "Virulent" and three each with one-half loop of "Avirulent," the organisms in every instance being suspended in one c.c. of broth. After eight days the rabbits were all killed by bleeding, and the agglutinative and bactericidal value of the serum determined in each case. Rabbits inoculated with the virulent culture furnished better serum than those inoculated with the avirulent, but the value in both agglutinative and bactericidal properties of the serum from the animals treated with the former was in no case more than two and one-half times that of the serum furnished by animals treated with the latter strain.[1]

These results were somewhat at variance with those of Haffkine, and still more so with those of F. Pfeiffer and Friedberger, who used dead cholera spirilla of different degrees of virulence. It was therefore decided to repeat the experiment, the virulence of the cultures being determined as before on the day of inoculation. The result was practically the same, the serum showing at the end of eight days that the virulent strain had in but one case given a serum of more than two and a quarter times the bactericidal value of that produced by the avirulent. In this one case the avirulent serum was between one-fourth and one-fifth as strong as the virulent serum. From the results of those experiments with intravenous injection of *living* organisms into rabbits we might apparently assume that the immunity produced is not directly proportional to the virulence of the inoculated organism.[2]

In the experiments performed with subcutaneous injection of the avirulent organism, both living and dead, the local reaction, as already mentioned, was much milder than with the use of the virulent germ; but there was always considerable local inflammation about the point of inoculation. Hence it appeared desirable to do away, if. possible, with the bacterial cells.

In this connection it is necessary to recall that a satisfactory protective against Asiatic cholera must probably contain substances which give rise to antitoxic as well as bactericidal bodies in the blood sera of the inoculated. A consideration of both these substances was thus necessary; and the problem of their extraction from the bodies of the bacteria was next investigated.

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[1] Tables giving the details of these and other experiments appear in full in Bull. No. 16, 1904, of the Bureau of Government Laboratories, Manila, P. I.

[2] These questions of the relation between immunity produced and virulence of culture used will be discussed in more detail in a later paper.

The toxin.—In epidemic cholera the symptoms often develop very rapidly after they have once begun, so that, usually within a few hours after their appearance, either death or a severe state of collapse has supervened. The cholera process cannot be satisfactorily explained except as the effect of an organic poison, and it therefore appears likely that in a very short space of time a considerable amount of toxic material is manufactured and liberated. Further, cultures made from the intestinal material during the period of most acute symptoms and compared with those taken 48 hours or more after the entrance of the stage of collapse show that in the latter condition there is usually a remarkable reduction in number of cholera spirilla and apparently a great increase in the number of other intestinal bacteria. If this clinical experience be compared with the observations made in the laboratory, namely, that in agar cultures of cholera spirilla the maximum growth at 37° C. is obtained in from 12 to 20 hours, and that after this time a rapid death of the spirilla takes place,[1] it may be inferred that possibly the stage of the most violent symptoms in human cholera corresponds with the period during which the rapid dying off of the spirilla occurs. It might also be possible that at the time of death of the spirilla the largest amount of toxic substance is set free.

The isolation of this specific substance, the existence of which was first argued by Koch, has been often attempted, by Hueppe among the first, and by many other investigators; but today it would appear that none of these authors was dealing with the specific cholera toxin alone. Pfeiffer failed to demonstrate any soluble toxin in young broth cultures, and only a ptomaine-like body in old broth cultures. He was able to show, however, a powerful toxic substance in killed fresh agar cultures. His work has been confirmed by others, among them Kolle and Wassermann, who show that the true cholera toxin exists as a constituent element of the bacterial cell. This poison becomes soluble only by the disintegration of the bodies of the bacteria, and the fluid culture is very unstable and soon destroyed. It is true, Metchnikoff and Roux profess to have demonstrated a soluble cholera toxin by its action through a collodion sac in the abdominal cavity of a guinea pig; they also isolated the toxin. But repetitions of their experiments by other observers have not been successful. On the other hand, the results of Buchner on cholera plasmin, in which the toxin was extracted by grinding and pressing the germ cells, support the intracellular hypothesis; and we may conclude that the most reliable evidence shows that the cholera spirillum does not produce a powerful soluble toxin, but that its toxin exists as an integral part of the cell, from which, through plasmolysis and digestion, it may be set free.

The ferments.—Another necessary consideration in preparing a cholera prophylactic was the action of the enzymes of the cholera spirillum, their effect upon the protoplasm of their own cells, and the effects of the digestive products thus formed. The cholera vibrio, under certain conditions, produces at least four of these enzymes, the diastatic (Bitter), the inverting

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[1] According to Gotschlich and Weigand, after two days only 10% (at the maximum), and after three days only one % (at the maximum) of the organisms present at the end of the first 24 hours were still alive.

(Fermi), the rennin-like (Schoffer), and the peptonizing (Bitter). The work of Gotschlich and Weigang and that of Conradi have shown that the rapid dying-off of cholera spirilla in agar cultures after 24 hours results from the action of degenerative products formed autolytically in the culture. This digestion is probably due to the peptonizing ferment.

PREPARATION OF THE PROPHYLACTIC.

With these two considerations in mind, (1) that the cholera toxin exists as an integral part of the bacterial cell, and, (2) that it is set free after the death of the organism, probably partly through the action of its own proteolytic enzyme, which is not destroyed at 60°, I endeavored to determine whether the other immunizing substances (agglutinin and bacteriolysin), as well as the toxin, could not be separated from the bodies of the bacteria by a process of autolytic digestion.

Accordingly, cholera spirilla from races known to possess good peptonizing powers were placed in an aqueous solution, carefully killed by heating, and digested at 37° C. It was then found that the cholera receptors were set free in the fluid in great abundance, a fact easily demonstrable after filtration, since such filtrates possessed the power of binding uni- and ambo-ceptors (agglutinins and bacteriolysins) in a cholera immune serum, as well as the ability of giving rise, after injection into rabbits, to the appearance of toxic symptoms, and in the case of the ultimate recovery of the rabbit, to the entrance of antitoxic, bactericidal, and agglutinative substances into the blood serum. Such a filtrate, therefore, immediately recommended itself as a trial substance for preventive inoculation. It was prepared in large quantities after the following manner:

The surfaces of large flat-sided flasks (after the pattern of Kolle), filled with agar, were sprayed with 20 hours' broth cultures of the organism, and the flasks put aside for 20 hours at 37° C.[1] The growth was then suspended in sterile water and placed in a sterile flask at 60° C. for from one to 24 hours. The mixture was afterwards put at 37° C. for from two to five days, and finally filtered through a Reichel candle.

In certain experiments the milky fluid overlying the sediment of the bacteria at the bottom of the flask was poured off and the latter crushed and subjected to hydraulic pressure of 600 atmospheres. Later the extracts from these pressed organisms and the original aqueous solution, previously decanted, were together passed through the filter.

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[1] Cultures of 20 hours' duration were always used, on account of the rapid death of the organisms in older cultures. Fresh beef was employed in the preparation of the media. The agar at the time of use had an alkalinity of – 1% to phenolphthalein. This gave a sufficient alkalinity to the aqueous suspensions of the organisms to bring about a favorable action of the proteolytic ferment.

EXPERIMENTS WITH THE PROPHYLACTIC.

INTRAVENOUS INOCULATION.

Prophylactic I.—Intravenous inoculation of four rabbits with from two to three c.c. of a fluid obtained in the above manner from the virulent strain, in which, however, the organisms were heated for only one hour at 60° C., caused the death of all the animals. It was thought advisable therefore to weaken this toxic action by a more prolonged heating of the organism, in order that bactericidal and agglutinative immunity might be studied in the inoculated animals.

Rabbit series No. 2.—For this series of rabbits, the suspension was placed at 60° C. for 24 hours, and digested for two days at 37° C., before passage through a Reichel filter. For the sake of comparison, both stems, "Virulent" and the "Avirulent," were treated in this way. One cubic centimeter of each filtrate represented the number of receptors obtained from two loops of the living organisms. The rabbits were of about the same average weight (1,500 grams) as those used in *Series No. 1.* Each animal was inoculated intravenously with one c.c. of the filtrate (equal to two loops). After eight days they were all killed by bleeding and the bactericidal and agglutinative values of their blood sera were determined.

With the intravenous injection into rabbits of one c.c. of virulent Prophylactic I, there were obtained sera showing an agglutinative value with the virulent stem of about 3.3 and 2.5 mg., and with the avirulent one of about 2.0 and 1.66 mg., and a bactericidal value against the former strain of about 0.09 and 0.08 mg. However, in the rabbits inoculated with the avirulent prophylactic the sera were not of nearly so great a value, showing an agglutinative worth of only about 10.0 to 3.3 mg., and a bactericidal one of 1.1 to 0.5 mg.

This series of experiments suggested that by the use of the method of autolytic digestion a better bactericidal immunity was obtainable with the virulent organism, and that the immunity acquired was within certain limits directly dependent upon the virulence of the stem used in the preparation of the virus. The value of the sera obtained from the animals inoculated with the virulent prophylactic also offered encouragement for a more extensive trial of this method with certain modifications.

Prophylactic II.—Therefore another quantity of the prophylactic was prepared, slight changes being introduced in the method. Twenty-hour agar cultures of the virulent organisms were suspended in sterile distilled water and the suspension divided into three portions, each being placed in a separate sterile flask and kept at 60° C. for 24 hours. The first portion was allowed to digest for two days, the second and third for five. All three were then filtered separately, after which the third was reheated at 60° C. for two hours. In the case of Prophylactic II twice as much distilled water was used in preparing the suspension of the agar cultures as in Prophylactic I. Hence, one c.c. of this filtrate contained only the number of receptors obtained from one loop of the living organisms, and possessed only one-half the strength of Prophylactic I.

Rabbit series No. 3.—Four rabbits were injected intravenously, each with 12 c.c. of this prophylactic, animals Nos. 86 and 87 receiving the portions digested for only two days, animal No. 88 that digested for five days, and animal No. 89 that reheated for two hours at 60° C. after five days' digestion; each animal received the number of receptors obtained from the digestion of 12 loops of the living organisms. After eight days the animals were killed by bleeding and the values of their sera estimated.

The results obtained in this series of experiments gave still greater encouragement for the method, since they showed that by a digestion of five days more receptors were set free from the bacterial cells in an aqueous solution than by one or two days. Animals Nos. 86 and 87, each receiving inoculations of the portions digested for two days, showed an agglutinative value of about 1.25 and 1.43 mg., and a bactericidal immunity of about 0.05 mg.; while animal No. 88, inoculated with the portion digested for five days, showed an agglutinative value of 1.1 mg. and a bactericidal value of about 0.04 mg. Results with No. 89 showed that reheating at 60° C. for two hours had destroyed to some extent the agglutinable substances of the bacteria, as evidenced by a marked loss of agglutinin in the serum, show only 1.6 mg. in value. The substances giving rise to the bactericidal qualities were apparently but little affected by the second heating, since the bactericidal value of the serum of this animal was between 0.04 and 0.05 mg., only a little poorer than that of animal No. 88 (0.04 mg.).

Prophylactic III and IV.—In order to determine whether more receptors are always set free in the fluid with longer digestion, two more portions of the prophylactic were prepared with both strains, one being digested for five, the other for two days at 37° C. In Prophylactic III sufficient sterile water was used for the suspension of the organisms to make one c.c. of it equal to the number of receptors obtained from two loops of the living organisms; in Prophylactic IV one c.c. of the suspension equaled one loop. Hence, in the inoculation of the rabbits in which Prophylactic IV was employed, double the amount of fluid was injected. The rabbits which received Prophylactic III, digested for five days, all furnished better sera than those injected with the virus of corresponding virulence of Prophylactic IV, digested for only two days. The best serum obtained from the injection of virulent Prophylactic IV was that of animal No. 256, which showed an agglutinative value of 1.4 mg. and a bactericidal one of 0.06 mg.; while the best obtained with Prophylactic III was from animal No. 192, showing an agglutinative limit of one mg. and a bactericidal value of 0.04 mg. In other words, these experiments indicate that even when corresponding amounts of the digested organisms in solution were injected, the animals receiving the more concentrated virus furnished the better serum. However, the differences in value of the sera are so slight that, unless constant, they might be explained by natural variation in the animals used. From these experiments it seemed that sufficient data had been obtained toward securing a good agglutinative and bactericidal serum by means of a protective prepared after this method, and that its success for intravenous injections was assured.

SUBCUTANEOUS INOCULATIONS.

The next step, then, was to discover what degree of immunity could be obtained when the prophylactic was injected subcutaneously. Accordingly, experiments were made with both strains, the inoculations being performed subdermally in rabbits. In such a series of experiments it was found that after the subcutaneous injection of five c.c. of the most favorable virus, sera were obtained with an agglutinative value of from 2.5 to 1.6 mg., and a bactericidal value of from 0.14 to 0.1 mg. These results were regarded as very favorable.

EXPERIMENTS WITH THE DRIED PROPHYLACTIC.

In several instances the prophylactic was evaporated in a vacuum at 38° C. and then pulverized, after which it was redissolved in normal salt solution, and injected into rabbits both intravenously and subcutaneously in varying amounts. While a distinct loss in the potency of the prophylactic thus treated is evident, nevertheless, a very good agglutinative serum and bactericidal immunity were obtained. Animal No. 167, inoculated intravenously with 10 mg. of the powder obtained from virulent Prophylactic II, produced, after eight days, a serum which agglutinated in dilutions of 10 mg., and showed a bactericidal reaction in dilutions of 0.25 mg. Animal No. 187, inoculated subcutaneously with five mg. of the powder obtained from virulent Prophylactic III, showed after one week a serum of a weak agglutinative value of 10 mg. and a bactericidal one of 1.6 mg.

STUDY OF THE LOCAL AND GENERAL REACTION FOLLOWING THE INOCULATION OF THE PROPHYLACTIC.

In general, it may be said that the animals apparently suffered little from the injection of the prophylactic, even when large amounts were employed. Usually they showed a rise of temperature of one, rarely two, degrees during the 36 hours immediately following the inoculation. A number of rabbits were treated subcutaneously with each separate lot of the prophylactic, for the sole purpose of observing the local reaction. Even when large amounts of the virulent prophylactic (five c.c.) were used for subcutaneous inoculations no suppuration ever occurred, and induration was rarely observed. Usually after 24 hours there was no trace of a local reaction visible. These animals showed a slight and transitory rise of temperature of one or two degrees.

As regards retention of immunity, it may be stated that several animals were killed from three to six months after inoculation, when an examination of their blood sera still demonstrated a high agglutinative and bactericidal reaction.

COMPARISON OF THE IMMUNITY PRODUCED BY THE VIRULENT AND THE AVIRULENT PROPHYLACTIC.

In comparing the immunity obtained by the use of the virulent and the avirulent prophylactic, we see that on the whole the results already referred to are borne out. It will be recalled that in one case the ratio of bactericidal immunity between the animals treated with the virulent prophylactic and those treated with the avirulent one varied between about 3½:1 and 12:1. In another series the sera obtained from the animals inoculated with the virulent prophylactic showed a bactericidal value from about 5½ to 12 times as great as that obtained from the injection of corresponding amounts of "Avirulent." In other cases the animals of the "Virulent" series showed sera from 6 to 15 times as great in value as those of the "Avirulent" ones. With subcutaneous inoculation the proportion is from 8 to 11 times as great; and with the dry prophylactic the value is from 1⅓ to 4 times as great. The results obtained with the dry prophylactic are certainly not so accurate as those with the fluid, on account of the manipulations to which the powder was subjected; and since they are not in accord with all the other numerous experiments, in which the liquid prophylactic was employed, they need not be considered in this comparative consideration. With this exception the results here reported are in harmony with those which have been obtained by other observers who have employed for inoculation strains of the killed organism of different virulence; namely, that the immunity obtained is within certain limits directly proportional to the virulence of the inoculated strain.

Upon comparing the immunity obtained by the intravenous injection of the prophylactic injection into rabbits with that produced in the same manner by the inoculation of the living organisms, we see that by the injection of one c.c. of the virulent prophylactic (representing the number of receptors obtained from two loops after two days' digestion), there is occasionally obtained a serum nearly equaling in bactericidal and agglutinative properties that produced by the intravenous injection of one-half a loop of the living virulent organisms. By a single intravenous injection of six c.c. of the prophylactic (obtained from 12 loops after five days' digestion), a serum of far greater value was produced, namely, one agglutinating in dilutions of 1:900 to 1:1000 (one mg.) and showing a bactericidal value as high as 1:24,000 (0.04). Therefore, by a judicious use of this method of autolytic digestion a means is offered us of producing by a single intravenous injection into rabbits a serum of greater bactericidal and agglutinative value than could be produced through the employment in the same manner of either the killed or the living organisms. It is known that an agglutinative value of one mg. and a bactericidal one of 0.04 mg. are not usually to be obtained by the single injection of cultures of either the killed or the living cholera vibrios, even when the inoculation is repeated.

EXPERIMENTS WITH THE PROPHYLACTIC ON GUINEA PIGS.

The following experiments show the effect of the prophylactic on guinea pigs, and also the comparative value of the protection furnished by the virulent prophylactic and that furnished by the injection of living organisms.

Guinea pig series No. 1.—From 6 to 12 animals were inoculated intraperitoneally and subcutaneously with varying amounts of Prophylactics I, II, III, and IV, and of dried Prophylactics II and III respectively, redissolved in normal salt solution. The dose varied from one to five c.c. From 7 to 10 days after the injection of the prophylactic, the animals received intraperitoneally either 5 or 10 times the fatal dose of the living virulent cholera strain. When the "Virulent" prophylactic was employed, the animals were invariably protected; but when the "Virulent" one was used in small amounts of one to two c.c., the guinea pigs sometimes succumbed to the subsequent injection of the living organisms. A few of the animals which received large amounts (five c.c.) of the virulent prophylactic intraperitoneally succumbed, evidently on account of its toxic effects. Upon autopsy no injection of the vessels or hemorrhages at the point of inoculation, such as are always found when death occurs from the inoculation of the living or the dead organisms, were observed. Neither were there any hemorrhages in the serous surfaces of the peritoneum. The most noticeable lesions in these cases consisted of a marked edema of the abdominal walls with some flakes of fibrin over the liver. Microscopically it was observed that an extensive desquamation of the epithelial cells had occurred. The contents of the abdominal cavity were sterile. It is to be noted that one to two c.c. of the thick material, consisting of the débris of the bacteria which accumulated at the bottom of the flask in the manufacture of the prophylactic, and remains behind on the filter in the form of an emulsion, after the prophylactic is passed through, when injected intraperitoneally, causes the death of guinea pigs, in which are found postmortem the most extensive local reactions at the point of inoculation and throughout the abdominal cavity, consisting of hemorrhagic areas, injection of the larger vessels, and corrosion of the tissues. These effects evidently are caused by toxic substances forming a constituent of the bacterial membrane not soluble in aqueous solution after autolytic digestion, and which have probably little to do with the production of the true immunity against the disease.

Guinea pig series No. 2.—Four large guinea pigs of about the same weight were chosen. Two were inoculated intraperitoneally with 1 to 15 loops of the living virulent organisms and two with five c.c. of the virulent Prophylactic IV. One of those which received the living organisms was very sick for 24 hours following the inoculation. After six days all the animals were reinoculated intraperitoneally with two loops of the living virulent strain. The two which had received the prophylactic previously, lived; the other two died.

Evidently the protection furnished the guinea pigs by the prophylactic was greater than that furnished by the previous injection of 1 to 15 loops of the living organisms, a supposition also borne out, and in a more striking way, by comparing the immunity obtained by the intravenous injection of the prophylactic into rabbits with that obtained by the injection of the living organisms. From the foregoing it is evident that the virulent prophylactic forms a reliable and certain means of protecting guinea pigs against the subsequent injection of multiple fatal doses of the cholera spirillum.

STUDY OF THE TOXIC ACTION OF THE PROPHYLACTIC.

As stated previously, in a few preliminary experiments made with the intravenous injection into rabbits of the virulent prophylactic, if the organisms had been killed by only a very brief period of heating and then allowed to digest themselves, the animals all succumbed to the inoculation. In order to study carefully the agglutinative and bactericidal value of the sera of the inoculated animals, an attempt was made to weaken the toxic action by prolonged heating at 60° C. This heating apparently had the desired effect, as the rabbits then usually survived the inoculations. In order to study this toxic action more closely, a prophylactic was prepared by killing the organisms within a very brief period, digesting at 37° C., grinding, submitting them to a pressure of about 600 atmospheres, and finally filtering through a Reichel candle. Varying quantities of the prophylactic were then injected intravenously into rabbits. The results show that two c.c. of virulent Prophylactic V prepared after this manner, when injected into rabbits, caused the death of these animals within 24 hours. Four and five c.c. injected in the same way produced death in a much shorter time. However, the animals sometimes recovered from the injection of one c.c., and were afterwards immune. With virulent Prophylactic VI, in which the filtration was performed with a coarser Berkefeld filter and the bacteria subjected to a more thorough crushing process, even one to two c.c. caused the death of rabbits. In the animals which succumbed to the inoculation, the kidneys were found, upon postmortem examination, to be swollen, and showed other evidences of parenchymatous nephritis. The mesenteric vessels were deeply injected, the liver congested and swollen. The lungs showed patches of congestion, hemorrhages, and in one or two cases small pneumonic areas. A few of the animals showed hemorrhages in the peritoneal surface of the small intestine.

Heating the organism at 60° C. evidently destroys most of the primary poison, or, at any rate, converts the toxin into toxoid, since it is necessary, in order to bring about the death of the guinea pigs, to inject intraperitoneally relatively large amounts (three to five c.c.) of the heated prophylactic. The presence of a toxoid would probably be more desirable in a human prophylactic than that of the unchanged toxin, *i. e.*, the toxoid, through the presence of its haptophore group (the toxophore group being lost) is still able to produce antitoxin in the inoculated body, without unfolding its general poisonous effect. Such a result we are able to obtain from the injection of our cholera prophylactic. It was found that three c.c. and even two c.c. of the serum of a rabbit (No. 422), which had previously been inoculated with two c.c. of the virulent heated Prophylactic V, protected other rabbits against two or three times the intravenous dose fatal for these animals; while two c.c. of human serum, obtained from an individual previously inoculated subcutaneously with three c.c. of virulent Prophylactic V, was capable of neutralizing about four times the fatal dose. In one case one-fifth c.c. of the serum of animal No. 423 (previously inoculated subcutaneously with five c.c. of virulent Prophylactic V) protected a rabbit against about four times the fatal dose of the toxin. In all of these experiments the prophylactic and the sera were mixed immediately before inoculation. The control rabbits without serum died; this being true also in cases in which equal amounts of normal serum were added to the prophylactic before injection. When smaller quantities of the serum were employed, the death of the animals always resulted, nor would two c.c. of the immune serum protect against any higher doses of the toxin. It is admitted that such antitoxic values of the sera, obtained from animals previously inoculated with the prophylactic, are not high; but it is hoped that better antitoxic properties can be produced when more improved methods are employed to extract the intracellular toxin.

In certain experiments recently performed, in which the digested bacteria were ground before filtration in a mortar with fine quartz sand and infusorial earth, a much greater toxic effect on the guinea pigs was observed, these animals dying from intraperitoneal injections of one-half to one c.c. of such a fluid. Judging from my own experience in this respect, it would appear that the most advantageous method for the extraction of the intracellular toxin of the cholera spirillum would be the one which MacFadyen has recently applied with the same end in view to the typhoid bacillus, *i. e.*, the bacteria are ground at the temperature of liquid air, the disintegration having occurred under conditions which precluded the possibility of chemical change. A combination of the method of autolytic digestion which I have described and the method of MacFadyen for the extraction of the toxin might perhaps furnish a more ideal prophylactic for Asiatic cholera, the mixture of the products of these methods being carefully heated at such temperature as to change the larger portion of the toxin into toxoid. My experiments show that with the modified and extracted toxin no local reaction, similar to that produced by the living or the killed cholera organisms, is obtained, even when sufficient amounts of the former are injected to cause death. The problem, therefore, which confronts us, is the extraction of the toxin in larger quantities.

From a few preliminary experiments, already performed with crude apparatus, it would seem that, with the appliances and the methods recommended by MacFadyen, such production should be more successful. My experiments throw little light on the nature of the structure of this intracellular toxin. That the haptophore group is identical in structure with that of the soluble toxins of diphtheria and tetanus bacilli would appear doubtful.

EFFECT PRODUCED UPON THE TOXIC ACTION OF THE PROPHYLACTIC BY HEAT OR BY ITS PRESERVATION WITH CERTAIN CHEMICALS.

One series of experiments was made[1] showing the effect of boiling upon the toxic action of the prophylactic when injected into rabbits. From these experiments it appeared that the toxic action was destroyed by a temperature of 100° C., since, after the prophylactic had been thoroughly boiled, neither an intravenous injection of one c.c. nor one of two c.c. caused the death of the rabbits inoculated with it; while control animals receiving one c.c. of the unboiled prophylactic always died. Control animals receiving two c.c. of peptone solution of the same specific gravity as the prophylactic were unaffected. Animals which received the boiled prophylactic were apparently but little disturbed by the inoculation, and their blood showed practically no agglutinative action with the virulent strain. Each successive heating of the prophylactic at 60° or over affects unfavorably the toxic as well as the agglutinative substances. However, by a careful heating at 60° for 15 minutes the toxin is apparently not entirely destroyed.

Several weeks' preservation of the prophylactic in chloretone at room temperature resulted in a loss of toxic power and a further change of the toxin into toxoid. After the preservation of the prophylactic for three months in chloretone neither one c.c. nor even two c.c. of it, when injected intravenously into rabbits, caused the death of these animals; though the injection of the latter amount produced illness with a rise of temperature of about two degrees. On comparing these results with those obtained by the use of fresh prophylactic, it is shown that formerly one-half c.c. of this prophylactic brought about death, and that one or two c.c. were always fatal. However, the agglutinable substance of the prophylactic, preserved for three months in chloretone, remained apparently unchanged in both its groups; since the same relative amounts of agglutinin were produced in the sera of the rabbits inoculated with the old prophylactic as were formed in those of the rabbits inoculated with the fresh one, values as high as one mg. being obtained. The bactericidal values of the sera of the animals were not investigated. There was no reason to suppose that any loss in bactericidal power would be found in these sera, as it is well known that the substances giving rise to the bacteriolysins are not so labile as those which produce the agglutinins.

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[1] In connection with the following experiments I wish to express my thanks to my assistant Mr. Charles B. Hare for much aid.

The preservation of the prophylactic with 0.5 carbolic for a long period of time gave practically the same effect as that produced by chloretone, a weakening of the toxic action, so that three c.c. of the carbolized prophylactic, as against one and two c.c. of the freshly prepared, were required to produce death in a rabbit. However, the substances giving rise to the agglutinins and bacteriolysins were apparently not unfavorably affected by this process.

On account of the unfavorable effects of these substances, it has usually been our practice so to handle the prophylactic in its manufacture that it is received from the filter into the sterile tubes, thus making unnecessary any further sterilization either by heat or by the addition of chemicals. With a prophylactic kept sterile and at the temperature of the ice-box, I have obtained very good results five months after its preparation. Of course, the toxic action becomes weaker after a short period of time, and this process gradually increases, owing to the still further change of toxin into toxoid.

HUMAN INOCULATIONS.

After studying the effects of the prophylactic upon animals, it was also desirable to ascertain its action upon human beings. With this end in view, a number of individuals have been injected from time to time with varying amounts (one to five c.c.) of the virulent prophylactic. The inoculations have been made deep into the muscles of the arm. In these cases the local reaction was never very marked. There was usually soreness on pressure in the region of the inoculation, lasting for about 24 hours, and occasionally a slight reddening of the overlying skin was observed. None of the patients have complained of much pain. No suppuration has ever been observed, and in fact it may be said that the local reaction is very slight. Following the inoculation there was generally a rise of temperature of from one to three degrees (Fahrenheit), which subsided in from 24 to 48 hours. Headache lasting for a few hours was occasionally complained of. Unfortunately for a further trial of the method, there has not been sufficient cholera present in the city or in the provinces during the past nine months to warrant the introduction of a general inoculation of the people against this disease; nor has there been any opportunity to observe, from an entirely practical standpoint, the immunity of the inoculated. The fact, therefore, that no cases of cholera have occurred among those receiving the prophylactic shows nothing in regard to the value of the method; since it is doubtful to what extent the inoculated have been exposed to the disease. However, it has been demonstrated that the blood sera of the inoculated individuals, both white and native, acquire protective substances. The sera of none of them, before inoculation, caused any agglutination of the virulent organism in dilutions of 1:20 (50 mg.) or any bactericidal action in dilutions of 1:50 (20 mg.). The blood was drawn from one of the veins of the arm one week after the inoculation and after the separation of the serum the value of the latter was determined. From these experiments it appears that three or four c.c. of the prophylactic furnished the best serum, namely, one having an agglutinative value of from 4 to 2.5 mg. and a bactericidal one of from 0.33 to 0.25 mg. These sera are ten times more potent than those obtained by Kolle from the subcutaneous injection of either the living or the killed cholera spirilla in human beings. Kolle's best sera showed a bactericidal value of from 3 mg. to 2.5 mg. They also show a higher value than is usually seen in the sera of human beings who have recovered from an attack of Asiatic cholera, which according to the investigation of R. Pfeiffer may be 10 mg. Therefore, we might presume that a good active immunity had been acquired against the disease by the use of this prophylactic. The antitoxic value of the sera has already been discussed. In case No. 1 two c.c. of the serum protected rabbits against four times the dose fatal for these animals.

We have seen that by the subcutaneous injection of the cholera prophylactic an excellent cholera immune serum can be obtained in human beings. However, the question naturally arises, whether these individuals are protected against intestinal infection with the cholera spirillum. In other words, are they really immune to the disease, Asiatic cholera? Experiments upon animals cannot satisfactorily answer this query. The earlier investigations of Brieger, Kitasato, Wassermann, Haffkine and others upon the point at issue, namely, whether animals could be rendered immune against intestinal infection with Asiatic cholera gave affirmative results. However the more recent work of Pfeiffer, Wassermann, and Sobernheim, demonstrated that immunity in animals against such infection was not to be obtained certainly by the ordinary methods of immunization then in vogue. Since animals are not naturally susceptible to intestinal infection, and since it is only through artificial means that such an infection may be produced in them, evidently the answer to our question can be given only by a practical observation of the individuals inoculated with the prophylactic during a severe and general epidemic of the disease. For this reason it was hoped that a more extensive practical demonstration of the value of the prophylactic could be given before an extended publication of the work was made.[1]

Since, however, the present report has been delayed nearly nine months and as it appears that there will be no greater opportunity in the near future for a more practical test of the prophylactic in these Islands than has already been experienced, it is thought inadvisable to defer for a longer period the publication of the experimental work.

It would seem, however, from the numerous statistics of Haffkine in India and the more recent work of Murata in Japan, that even by the injection of a small amount of the killed organisms a certain degree of immunity against the natural mode of infection is acquired. Therefore, judging from what has already been said, it is probable that by the use of our prophylactic human beings may acquire a good active immunity against the disease.

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[1] The results of the experimental work were presented to the Manila Medical Society at the meeting of September 7, 1903.

CONCLUSIONS.

1. By the autolytic digestion of carefully killed cholera spirilla in an aqueous fluid the receptors become separated from the bacterial cells and may be filtered off in solution.
2. The injection of these free receptors into both man and animals furnishes a means of producing high bactericidal and agglutinative blood sera. The antitoxic value of these sera is however moderate.
3. The subcutaneous injection into man of such free receptors is not only free from any danger, but produces practically no local disturbance and only a slight general reaction.
4. Hence the method is a practicable one for producing a cholera-immune serum in man.
5. It is highly desirable that this cholera prophylactic be given a thorough practical test.
6. It is possible that by the application to the pest bacillus of a slight modification of this method a more satisfactory prophylactic against bubonic plague could be obtained.

Experiments with this end in view have already been commenced in the Biological Laboratory.

The consideration of the comparative results in immunity obtained with the inoculation of the virulent and the avirulent living organisms and with the prophylactics of different virulence will be considered in another paper.